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Spore Viability Under Different Storage Conditions in Four Rupicolous *Asplenium* L. Taxa

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ABSTRACT.—Spore germination of four rupicolous taxa of *Asplenium* (*A. adiantum-nigrum.* var. *adiantum-nigrum*, *A. adiantum.nigrum.* var. *silesiacum*, *A. septentrionale* subsp. *septentrionale* and *A. ruta-muraria.* subsp. *ruta-muraria*) was determined after 1, 6, and 12 months of storage in Eppendorf tubes (dry storage) or on agar plates (wet storage) at –20, 5 and 20°C. In general, technique and temperature factors and the moisture-temperature interaction, had a significant effect on germination percentage. In all cases, except for *A. ruta-muraria*, germination percentage was maintained in wet and dry storage, but in the dry storage method percent germination was higher. These results indicate some capacity of *Asplenium* spores to withstand desiccation, and that ecological requirements of species may influence spore viability and should be taken into account when designing spore conservation programs. Spores of *A. ruta-muraria* yielded better results in wet storage. In dry storage its response was different from that of the other three taxa. Wet storage at –20°C killed all or most spores of all taxa.

Interest in the conservation of pteridophyte spores has become evident in recent decades, because they are easy to obtain, can be stored in large quantities, and can germinate rapidly in simple media (Dyer, 1979). Spores are of interest not only in *ex situ* conservation programs, but also, as Page *et al.* (1992) show, in taxonomic studies in the broadest sense, and as a commercial source in horticulture. However, in contrast to seed conservation (Baskin and Baskin, 2001 and references therein), little is known about the factors that affect spore viability during storage.

Lloyd & Klekowski (1970) calculated the variation in viability of chlorophyllous (green) and non-chlorphyllous spores over storage periods of 2 months to 3 years, noting the marked contrast between *Equisetum* (12 to 24 days viability) and *Asplenium* (up to 48 years).

The conditions under which spores are stored have a notable impact on their viability. Generally, to avoid deterioration, they are stored in dry, ambient or low temperatures, although in some cases this has resulted in loss of viability (Beri and Bir, 1993; Camloh, 1999). Another option that has been tried is storage of spores in a hydrated state (Lindsay *et al.*, 1992), analogous to conditions prevailing in natural spore banks in which spores of some species can remain viable for long periods (Lindsay and Dyer, 1990). It has been

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observed that this type of storage may be more effective than dry storage for certain species.

Pteridophyte spores may remain viable, in a metabolically inactive state, when conditions are not adequate for germination (Page, 1979). The length of time over which spores can maintain viability varies enormously from species to species (Miller, 1968) and it has been shown that other characters, such as spore age (Raghavan, 1989 and references therein), ploidy level (Kott & Peterson, 1974; Kott & Britton, 1982), and the presence of chlorophyll are influential. Even though they can survive desiccation (Lloyd and Klekowsky, 1970; Lebkuecher, 1997), chlorophyllous spores have generally limited viability, compared with pteridophytes with much longer-lived non-chlorophyllous spores.

Page *et al.* (1992) point out the need to investigate storage conditions that guarantee the maintenance of spore viability for the longest possible time, their genetic integrity, and their developmental capacity. Having available collections of adequately stored spores is of interest in order to avoid the loss of species in nature, in the case of threatened species, while at the same time offering the possibility of having subsequent developmental phases with which to investigate other aspects of the biology of the species.

In the present study, various storage conditions were tested on the spores of four *Asplenium* taxa: *A. septentrionale* (L.) Hoffm. subsp. *septentrionale*, *A. ruta-muraria* L. subsp. *ruta-muraria*, *A. adiantum-nigrum* L. var. *adiantum-nigrum*, and *A. adiantum-nigrum* var. *silesiacum* (Milde) Viane & Riechstein. These taxa constitute a homogeneous biogeographical and ecological group. They are circumboreal species (Pichi Sermolli *et al.*, 1998) orophyllous, rupicolous, and all are tetraploids. *Asplenium septentrionale* subsp. *septentrionale* is an autotetraploid derived from subspecies *caucasicum* Fraser-Jenkins & Lovis (Lovis, 1964) and is widely distributed throughout Eurasia as well as disjunctly in North Africa and on the Pacific coast of North America, where it preferentially inhabits acid substrates. *Asplenium ruta-muraria* subsp. *ruta-muraria* is also an autotetraploid but derived from subspecies *dolomiticum* Lovis & Reichst. (Lovis, 1964) and is distributed across a broad belt in the northern hemisphere, in Europe, Asia and America, having its southern limit in North Africa. It prefers basic substrates. Finally, *A. adiantum-nigrum* is an allotetraploid arising from crossing and subsequent chromosomal duplication of *A. cuneifolium* Viv. and *A. onopteris* L. (Shivas, 1969). It has a wide range throughout Europe, Macaronesia, Asia, Africa, North America and Australia, where it colonizes cracks and fissures preferentially in siliceous rocks. Two varieties are recognized: the typical variety described above, and the variety *silesiacum* (serpentinicolous ecotype), of ultrabasic substrates in northern and western Europe (Salvo, 1990).

Different degrees of hydration (wet and dry) have been combined with different temperature regimes in order to analyse the percentage germination after varying periods of storage. This same methodology has been used in a previous study (Quintanilla *et al.*, 2002) of a group of relict Macaronesian species that inhabit forest floors. These authors aimed to optimise the method

TABLE 1. Location of populations studied, collector and date of collection.

Taxa	Location	Altitude	Coordinates UTM	Collection data	Collector(s)
A. septentrionale	Madrid. Manzanares El Real. La Pedriza	1300	30TVL2310	July 2000	C. F. Aragón & R. G. Camacho
A. adiantum-nigrum var. *adiantum-nigrum*	A Coruña. A Baña.	225	29TNH2153	October 2000	L. G. Quintanilla
A. adiantum-nigrum var. *silesiacum*	A Coruña. Sierra de la Capelada. Chao do Monte	280	29TNJ8139	October 2000	J. Amigo & L. G. Quintanilla
A. ruta-muraria	Guadalajara. Somolinos.	1000	30TVL9263	July 2000	E. Pangua & S. Pajarón

of storage of viable spores as part of a conservation strategy. Although the taxa included in this study present no problem from a conservation point of view, our objective was to establish whether the optimal spore storage method varies among the taxa.

MATERIAL AND METHODS

Spores were obtained from populations of each taxon (Table 1). Fertile fronds of 15 sporophytes were collected per population and transported to the laboratory where they were washed under tap water and pressed for two weeks until the spores were released. Each sample was obtained from a mixture of spores from all sporophytes gathered in each population.

The spores of these four taxa were subjected to different storage conditions in which different degrees of hydration (wet and dry techniques) and temperatures were combined. With the wet technique, spores were sown directly on a mineral agar medium (Dyer, 1979) that had been sterilized in an autoclave at 20 atm and 125°C for 20 minutes, on sterile plastic Petri dishes (5.5 cm diameter), which were sealed with Parafilm (American National Can, Chicago) to avoid desiccation. To prevent contamination, the antifungal agent Nystatin (100 U ml^{-1}) was added to the culture medium after autoclaving and also, in all sowings the spore samples were passed through two layers of lens cleaning tissue (Whatman International Ltd. Maidstone, n° 2105841) to eliminate impurities, remains of sporangial walls, etc. With the dry technique, spores were kept in Eppendorf tubes until germination tests were carried out at which time they were plated out as above. The dishes and tubes were stored at temperatures of 20, 5 and −20°C. They were kept in the dark by wrapping them in aluminium foil to avoid germination during the storage period.

Germination tests were carried out after 1, 6 and 12 months of storage. All dishes were incubated for 30 days in a culture chamber at 21°C and 30 μmol m^{-2} s^{-1} intensity of photonic flow, with a 16 h light: 8 h dark photoperiod. Four replicates were incubated for each combination of technique and temperature

and the percentage germination was assessed after 30 days. This same germination test was carried out before storage to establish a control group. Germination was considered to have occurred if the spore wall was broken and the first rhizoid had emerged. Germination rate (%) was calculated on the basis of a count of 100 randomly chosen spores from each dish.

To determine the effects of technique (wet and dry) and temperature (20, 5 and –20°C) on germination rate, the percentages were arcsine-transformed and their means compared by a two-way analysis of variance (Zar, 1999). The analyses were repeated for 1, 6 and 12 months of storage. The multiple comparisons among means for the identification of homogeneous groups, wherein the effect of a factor was significant ($p < 0.05$), were made using the Tukey test ($p < 0.05$). All analyses were done with the SPSS statistical program (1999).

Results

As in the majority of pteridophyte species, spores of the taxa studied required the presence of light to germinate. The effect of technique and temperature factors, and their interaction, upon spore germination was statistically significant in all cases, except for the hydration factor after one month of storage in the case of *A. ruta-muraria* (F = 0.059; Table 2). The existence of a significant interaction between factors implies that the effect of each is different for each level. Thus, multiple comparisons between media were made for each possible combination of hydration and temperature.

The response of *A. adiantum-nigrum* var. *adiantum-nigrum*, *A. adiantum-nigrum var. silesiacum* and *A. septentrionale* to the different storage conditions was similar (Fig. 1A, B and C), whereas those of *A. ruta-muraria* were more variable (Fig. 1D). In general, high percentages of germination were found in the first three taxa, irrespective of the storage conditions, except for wet storage at –20°C, in which case only a small percentage of spores of these three taxa germinated after the first month. In these three cases, dry storage was fairly effective, although there was a slight decrease in the percentage of viable spores after 12 months of storage when kept at –20°C (Fig. 1A, B and C). Dry storage at 5°C (Table 3) was significantly higher ($p < 0.05$) than for dry-storage at 20 and –20°C, in the case of *A. adiantum-nigrum* var. *silesiacum*, and at –20°C for *A. septentrionale*, after 12 months storage (see Table 3).

With respect to *A. ruta-muraria*, the best results were obtained with wet storage at 20 and 5°C; no spores germinated at –20°C. Percentages achieved with the dry technique were generally lower than under humid conditions and lower than in the other taxa, except for the results after 6 months' storage at 5 and –20°C (Fig. 1D), where similar percentages were obtained to those with wet storage (Table 3).

Discussion

Our results indicate that for the taxa studied, except *A. ruta-muraria*, storage under any of the tested conditions allowed relatively high percentages of viable spores, except with wet storage at –20°C. Under those conditions there

TABLE 2. Levels of significance of the effects of technique and temperature factors on percentage spore germination after 1, 6 and 12 months of storage. MS, mean squared; df, degrees of freedom; *, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.001$.

			Storage time					
			1 month		6 months		12 months	
Taxa	Source of variation	d.f.	MS	*F*	MS	*F*	MS	*F*
A. septentrionale	Technique	1	2,303,118	91,188***	1,583,659	77,284***	849,024	23,600***
	Temperature	2	2,167,125	85,803***	3,168,146	154,609***	3,727,413	115,463***
	Technique × Temperature	2	1,945,107	77,013***	1,829,107	89,262***	1,503,217	46,565***
	Error	18	25,257	...	20,491	...	32,282	...
A. adiantum-nigrum var. adiantum nigrum	Technique	1	1,983,658	88,865***	2,361,546	214,497***	1,960,820	207,121***
	Temperature	2	2,513,572	112,603***	3,809,133	345,977***	3,316,035	350,271***
	Technique × Temperature	2	1,824,572	81,737***	2,859,518	259,725***	2,600,584	274,695***
	Error	18	22,322	...	11,010	...	9,467	...
A. adiantum-nigrum var. silesiacum	Technique	1	2,963,416	75,106***	3,358,753	126,386***	2,335,769	207,865***
	Temperature	2	2,506,259	64,104***	2,917,486	109,782***	2,674,307	237,922***
	Technique × Temperature	2	1,605,239	41,058***	1,899,082	71,460***	1,775,800	158,032***
	Error	18	39,097	...	26,575	...	11,237	...
A. ruta-muraria	Technique	1	15,918	0.059 ns	455,188	8,684**	2,263,204	22,964***
	Temperature	2	1,977,344	7,371**	1,941,044	37,032***	3,208,584	32,557***
	Technique × Temperature	2	1,305,054	4,865*	2,477,879	42,274***	1,491,976	15,139***
	Error	18	268,264	...	52,415	...	98,553	...

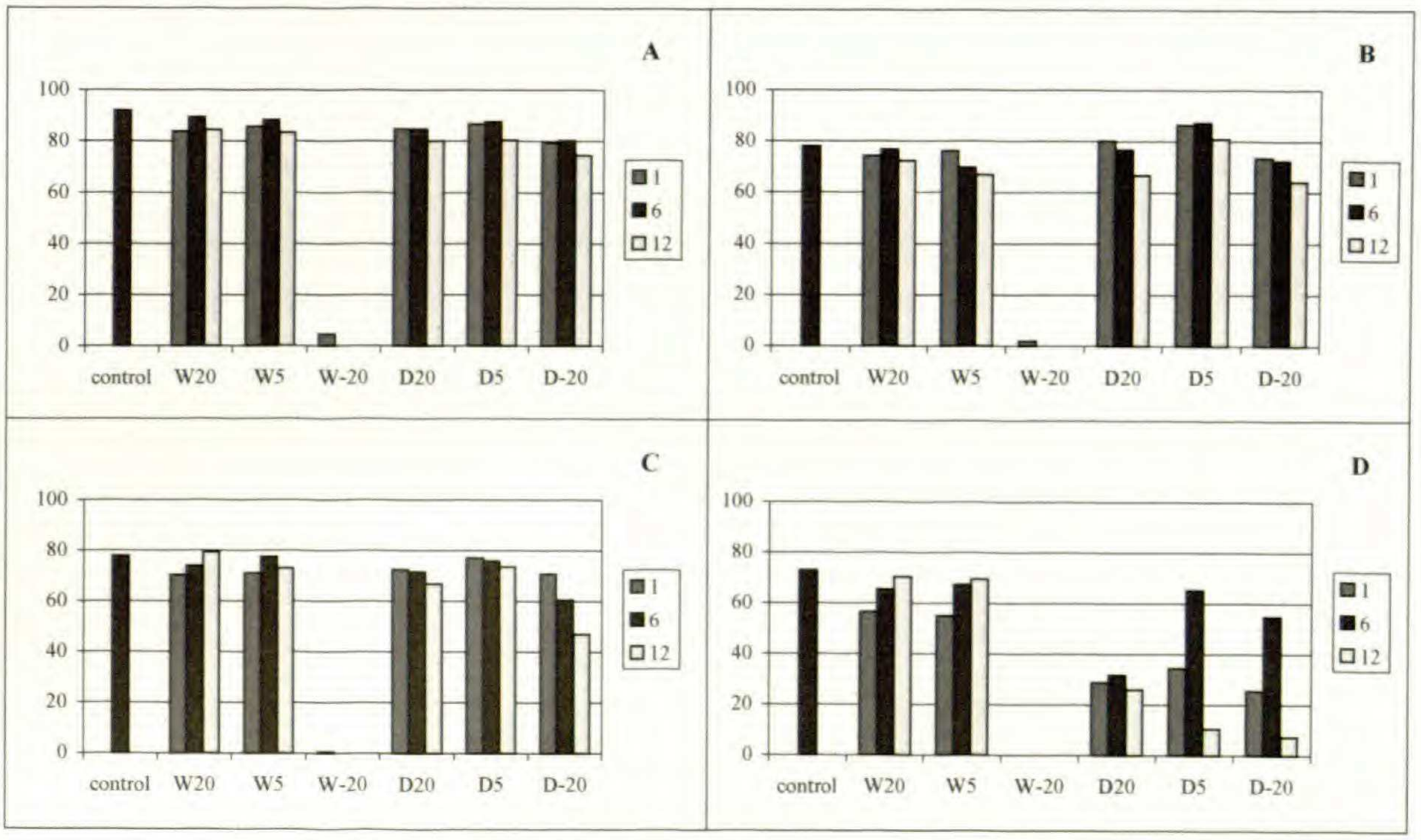

FIG. 1. Germination percentage after 1, 6, and 12 months' storage with different techniques (W, wet; D, dry) at temperatures of 20°, 5°, and –20°C. (A) *Asplenium adiantum-nigrum* var. *adiantum-nigrum*; (B) *A. adiantum-nigrum* var. *silesiacum*; (C) *A. septentrionale*; (D) *A. ruta-muraria*.

was practically no germination after one month of storage. This combination of hydration and temperature was generally inefficient at maintaining spore viability, confirming observations under identical conditions by Quintanilla *et al.* (2002) on relict forest species. This implies that this combination is not efficient irrespective of species ecology. Pangua *et al.* (1999) also noted a decrease in germination for spores of *Cryptogramma crispa* (L.) R. Br. kept in the wet at –18°C. Germination percentage in that species varied among populations. In this species wet spores subjected to a temperature of 70°C yielded higher germination percentages than did dry spores when subjected to the same temperatures after 24 h of treatment (Simpson and Dyer, 1999). Given that a dry –20°C treatment did not result in such drastic reduction in germination, it is obvious that previous hydration renders the spores more sensitive to freezing.

Hill (1971) showed that spores of *Adiantum pedatum* L. and *Thelypteris palustris* Schott, after a month of freezing in liquid medium, had higher percentages of germination for the characteristic periods of time than when spores were kept at ambient temperature. Although it has not been shown that longer preservation times yielded the same results, it nevertheless appears that these spores may require chilling in order to germinate. Hill (1971) did not specify whether the spores were frozen immediately after their inclusion in the medium or if there was a time of imbibition.

Wet storage at 20 and 5°C maintained the viability of a large number of spores of all taxa studied and hence represents an effective method of storage.

TABLE 3. Percentage germination (mean ± standard error) of spores without previous storage (control) and after 1, 6, and 12 months storage, with wet (W) and dry (D) techniques at temperatures of 20°, 5° and –20°C. The vertical lines indicate those groups with homogenous means, between which there are no significant differences.

		Storage time					
		1 month		6 months		12 months	
Taxa	Control	Germination	Treatment	Germination	Treatment	Germination	Treatment
A. septentrionale	77.8 ± 3.1	0.5 ± 0.5	W –20	0.0 ± 0.0	W –20	0.0 ± 0.0	W –20
		70.2 ± 1.5	W 20	60.5 ± 6.8	D –20	47.0 ±8.7	D –20
		70.7 ± 5.8	D –20	71.5 ± 3.6	D 20	66.7 ± 5.7	D 20
		71.0 ± 5.5	W 5	74.0 ± 1.4	W 20	73.0 ± 1.6	W 5
		72.5 ± 4.0	D 20	76.0 ± 3.4	D 5	73.5 ± 4.4	D 5
		77.0 ± 3.1	D 5	77.5 ± 2.6	W 5	79.2 ± 1.7	W 20
A. adiantum-nigrum var. *adiantum nigrum*	92.0 ± 1.1	4.5 ± 1.9	W –20	0.0 ± 0.0	W –20	0.0 ± 0.0	W –20
		79.5 ± 4.1	D –20	79.7 ± 3.1	D –20	74.5 ± 1.6	D –20
		84.0 ± 0.7	W 20	84.7 ± 1.8	D 20	80.0 ± 2.0	D 20
		84.7 ± 2.4	D 20	87.7 ± 2.9	D 5	80.5 ± 3.0	D 5
		85.7 ± 2.6	W 5	88.2 ± 1.0	W 5	83.2 ± 2.3	W 5
		86.7 ± 1.1	D 5	89.2 ± 1.5	W 20	84.5 ± 2.1	W 20
A. adiantum-nigrum var. *silesiacum*	79.0 ± 0.6	2.0 ± 1.7	W –20	0.0 ± 0.0	W –20	0.0 ± 0.0	W –20
		74.2 ± 3.5	W 20	69.7 ± 6.4	W 5	64.2 ± 1.2	D –20
		73.5 ± 7.2	D –20	72.5 ± 3.7	D –20	66.7 ± 2.1	D 20
		76.2 ± 2.8	W 5	76.7 ± 3.9	D 20	67.0 ± 2.8	W 5
		80.2 ± 2.3	D 20	76.7 ± 3.6	W 20	72.5 ± 2.6	W 20
		86.2 ± 2.5	D 5	87.2 ± 2.7	D 5	81.0 ± 4.1	D 5
A. ruta-muraria	73.0 ± 2.6	0.0 ± 0.0	W –20	0.0 ± 0.0	W –20	0.0 ± 0.0	W –20
		25.5 ± 17.5	D –20	31.7 ± 11.5	D 20	7.2 ± 4.7	D –20
		28.7 ± 6.1	D 20	54.7 ± 6.8	D –20	10.5 ± 7.4	D 5
		34.5 ± 17.1	D 5	65.2 ± 3.3	D 5	26.0 ± 10.7	D 20
		55.0 ± 10.4	W 5	65.5 ± 1.0	W 20	69.5 ± 3.2	W 5
		56.5 ± 13.2	W 20	67.2 ± 5.1	W 5	70.5 ± 1.8	W 20

Lindsay *et al.* (1992) studied the response to spore hydration of four species with non-chlorophyllous spores and one with chlorophyllous spores, all of which were hygrophilous. Fully hydrated spores were capable of germinating at ambient temperature after two years of storage at 20°C at much higher percentages than those preserved dry but under otherwise identical conditions. Other hygrophilous species, such as *Woodwardia radicans* (L.) Smith and *Culcita macrocarpa* C. Presl., show a marked sensitivity to desiccation, such that only those spores that had been maintained in a wet medium germinated after 12 months' storage, 60% and 84% respectively, compared with those kept in the dry, 1% and 0% respectively, at the same temperature (Quintanilla *et al.*, 2002). These results are interesting because spores of natural spore banks would be in a wet state (Page *et al.*, 1992), especially those from species that inhabit places where the soil is very wet throughout the entire year.

Dyer and Lindsay (1992) have shown the persistent presence of *A. adiantum-nigrum*, *A. ruta-muraria* and *A. septentrionale* (L.) Hoffm. in British spore banks. However, in the latter two strictly rupicolous species, the presence of spores in these banks is of low importance, because, although the gametophyte is already established, new sporophytes cannot be established, possibly due to a problem of competition with other species. Furthermore, these two species require a minimum temperature for germination (Young, 1985; Pangua *et al.*, 1994; Dyer and Lindsay, 1996), which they may encounter within cracks or other protected places. Nevertheless, they can tolerate temperatures of up to 70°C for at least 24 h (Simpson and Dyer, 1999). These results may represent an adaptive efficiency in these species for the media they inhabit – exposed rocks with large temperature differences throughout the day and the year.

In our study, for *A. septentrionale*, *A. adiantum-nigrum* var. *adiantum-nigrum* and *A. adiantum-nigrum* var. *silesiacum* dry storage gave results similar to those with wet storage, although perhaps longer-term storage would have revealed greater differences. Nevertheless, in light of the results, it appears that these rupicolous taxa, have a relatively high capacity to withstand desiccation. Therefore it appears that ecological requirements of species can indeed result in taxa specific adaptations in terms of spore viability, although it must be born in mind that few species have been studied and considerable variability may exist in this respect.

In *A. ruta-muraria* wet storage, with the general exception of –20°C for all the taxa studied, was significantly more effective than dry in maintaining spore viability. Results obtained at 5 and 20°C are essentially the same, showing a germination capacity that increased slightly with time of storage. However, the response obtained with dry storage at different sampling times is difficult to explain. Only after 6 months of storage at 5°C was the germination comparable to that of wet storage. These results might be explainable by a need to go through a cold period before germination. For *A. ruta-muraria*, despite not being a hygrophilic species, the most suitable preservation method is wet at 5 or 20°C.

In *Polystichum setiferum* (Forsskål) Woynar and *Athyrium filix-femina* (L.) Roth, typical of wet woodlands, it has been observed that dry storage at 4°C results in increased spore viability after 12 and 24 months, respectively, whereas at 20°C, spore viability is practically lost (Lindsay and Dyer, in Simpson and Dyer, 1999). Spores of *Cyathea delgadii* Sternb. (Simabukuro *et al.*, 1998) and *Pteridium aquilinum* (L.) Kuhn remain viable for years after dry storage at 4°C (Ashcroft and Sheffield, 2000), for which reason the authors have proposed the routine use of this storage technique and temperature. Some species with chlorophyllous spores, such as those of *Osmunda*, also retain their viability after years of dry storage at temperatures of 2° and 6°C (Stokey, 1951). In our case, therefore, it appears that the dry technique would be a good option for the conservation of the spores since, although the two techniques have yielded favourable results, the dry technique has some advantages, such as saving space, time and materials. The ideal storage temperature, in this case, would be 5°C, bearing in mind the results of our experiments, where the spores kept dry at this temperature had somewhat higher germination percentages in *A. adiantum-nigrum* var. *adiantum-nigrum*, and significantly higher percentages in *A. adiantum-nigrum* var. *silesiacum* and *A. septentrionale*.

Recent studies (Agrawall *et al.*, 1993; Pence, 2000) have demonstrated the effectiveness of preservation of dried chlorophyllous and non-chlorophyllous spores at –196°C in liquid nitrogen. Pence (2000) observed germination rates of spores of *A. ruta-muraria* stored under these conditions that were similar to that of the control population. These results may imply that imbibed spores are affected by very low temperatures, but that keeping them dry is a good conservation technique.

The time of storage and the processes of sterilization bring about alterations in germination and subsequent development of the gametophytes. Smith and Robinson (1975) studied germination of *Polypodium vulgare* L. using spores dry-stored at 4°C for 7 years. They observed a decrease in germination and an increase in the proportion of abnormal gametophytes. Similar results were obtained by Beri and Bir (1993) for *Pteris vittata* L., stored at room temperature for 100 days; spores lost germination capacity in association with total loss of sugars, amino acids and proteins. Camloh (1999) observed in *Platycerium bifurcatum* (Cav.) C. Chr. that sterilized spores lost viability and that with age there were fewer and shorter rhizoids. It would be interesting, in addition to germination studies for conservation, to study the impact on the development of the gametophyte. In our work, although we have not carried out a thorough post-germination study, the plates used in the various experiments remained in culture chambers at 20°C for 6 months and the gametophytes appeared to develop normally. This suggests that storage time may not affect the subsequent development of the prothalli.

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